EXHIBIT B

VH Chain Variable Region Binding Studies

US Application No. 10/579,290

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Materials and Methods

SAM-6 heavy chain variable region (V_H) alone was prepared using the following materials and methods: 1L culture flasks, 50ml Falcon tubes, Petri dishes, Eppendorf Biophotometer or equivalent to read OD_{600} and 280nm, 1.5ml Eppendorf tubes, LB amp agar plates, Bio-Rad 1ml spin columns, Vacuum Manifold, Suspension mixer.

Expression

For expression of SAM-6 heavy chain variable region (V_H) protein, fresh transformations of nucleic acid into JM109 were prepared by inoculating 10ml Super Broth (SB) amp medium with a single colony and grow at 33°C with shaking approximately 150rpm overnight, then dilute overnight 1:10 in SB. Record the culture OD600 (1ml as the blank SB) and then dilute 100µl of the culture plus 900µl SB. Grow culture until it reaches an OD600 of ~4.000. Take 1ml aliquot as the 0hour sample and store at 4^0C for later analysis. Shift the cultures to another incubator shaker set at 42^0C and continue shaking at approximately 150rpm for 4h. Check progress of induction periodically after 3 hours, once OD has stabilized induction is complete, record the culture OD600 (1ml as the blank) and then dilute 100µl of the culture plus 900µl terrific broth. Take 1ml aliquot at 4hours post-induction, transfer cultures to 50ml Polycarbonate Falcon Tubes (BD). Pellet down (spin at 4000g, 4^0C for 20mins) the balance of the induced culture for protein extraction and purification, and perform SDS-PAGE (Coomassie) and α-FLAG Western on the whole cell lysate aliquots and on the purified proteins.

VH Chain variable region isolation

Heavy chain variable region (V_H) protein was then extracted by lysing the cell pellet in 2ml BugBuster Master Mix (Novagen) and mixing on a suspension mixer for 20 minutes. The solution was then made to 6M Urea and gently mixed for a further 10 minutes, followed by addition of 600µl of Ni-NTA resin (QIAGEN) and mixing the solution on a suspension mixer for 60 minutes. The protein resin solution was then collected into a 1.0ml disposable spin column (Bio-Rad) under vacuum (10 in Hg) trapping the resin and bound His tagged scFvs in

the column, washed with 10ml of IMAC wash buffer, and then bound proteins eluted with 5ml of IMAC elution buffer.

Buffer exchange with eluted proteins was performed with Amicon Ultra-15 devices as follows: Wash devices with buffer to remove preservative on the membranes, usually glycerol (not essential but recommended). Place sample into filter compartment and make to 15ml with Hepes Buffered Saline (HBS), pH7.3 spin at 4000rpm (4°C) for 30 minutes (samples spun at 4°C will take longer than those spun at RT). Once sample is at ~1.0ml discard filtrate and add a further 14ml of HBS, pH7.3, to filter compartment and spin again. Recover protein sample and measure concentration at 280nm with Eppendorf BioPhotometer using BSA standard curve.

VH Chain variable region binding analysis

FACS analysis of heavy chain variable region (V_H) protein was performed as follows: 2 x 10^5 cells/ml (per sample) used in ice cold FACS buffer in 1.5ml eppendorf tube and let it sit on ice for 30 mins; for the control cells only, used 4 x 10^5 cells/ml. Centrifuge the tubes containing cells for 5 mins at 500g at 4°C, discard the supernatant, add the primary antibody at neat concentration (total volume 50µl), and leave on ice for 30 mins in the dark. Add 1ml ice cold FACS buffer (30 FACS buffer has 120µl, 2mM EDTA (stock 500mM), 300µl of 1% FBS HI, and 1xPBS, pH7.4, 30ml), and centrifuge for 5 mins at 500g at 4°C, add 2µl of the 2° antibody in 1/20 dilution in ice cold FACS buffer (total volume 50µl), leave on ice for 30mins in the dark. Add 1ml ice cold FACS buffer, centrifuge for 5 mins at 500g at 4°C, resuspend the cells in 200µl of ice cold FACS buffer (for the control cells only tube resuspend in 400µl). Filter through a nylon filter and transfer the cells to 5ml round bottom FACS tubes (check if there is any cell clumps), and just before analyzing add 1µl of Propidium Iodide (1mg/ml). Mix samples with a vortex mixer, and analyze the samples in the FACS machine. The data was analyzed with Win MDI program. Cell lines used were HeLa and HDFa cell lines.

FACS buffer was stored on ice. Propidium Iodide (Sigma, 1mg/ml), Polyclonal rabbit Anti-Human IgM/FITC (Dako, Lot no: 00051504), Anti Flag M2 Monoclonal FITC (Sigma, Lot No: 105K62091), Percivia SM-6 (+ J chain) clone 450 were all stored at 4°C.

Data Analysis

The results are illustrated in **Figure A**. In brief, in the top row, 1st Panel- scFv negative controls, 2nd Panel -SM6 2.7A scFv (black line), and 3rd Panel -SM6 opti scFv(black line). In

the bottom row, 1st Panel- SM6 VH alone (black line), 2nd Panel –anti-IgM negative control, and 3rd Panel -SM6 IgM 450 (black line).

In **Figure A**, the first (top) row there are two examples of SAM-6 single-chains (VH + VL). The SAM-6 2.7 contains the VH and VL using lambda light chain. The SAM-6opti contains the VH and VL using kappa light chain. The next (bottom) row has the VH domain only and exhibits similar binding to cells. The last panel (right) has the SAM-6 binding.

The first (left) panel on the second row shows binding of the SAM-6 VH alone to Hela cells. Thus, binding to SAM-6 target can be conferred by the VH alone of SAM-6.

Figure A Shows that V_H alone (without VL chain) binds to Hela cells.